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Suppression of NF-κB pathway by crocetin contributes to attenuation of lipopolysaccharide-induced acute lung injury in mice

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ABSTRACT

Crocetin, a carotenoid compound, has been shown to reduce expression of inflammation and inhibit the production of reactive oxygen species. In the present study, the effect of crocetin on acute lung injury induced by lipopolysaccharide (LPS) was investigated in vivo. In the mouse model, pretreatment with crocetin at dosages of 50 and 100 mg/kg reduced the LPS-induced lung oedema and histological changes, increased LPS-impaired superoxide dismutase (SOD) activity, and decreased lung myeloperoxidase (MPO) activity. Furthermore, treatment with crocetin significantly attenuated LPS-induced mRNA and the protein expressions of interleukin-6 (IL-6), macrophage chemoattractant protein-1 (MCP-1), and tumour necrosis factor-α (TNF-α) in lung tissue. In addition, crocetin at different dosages reduced phospho-IκB expression and NF-κB activity in LPS-induced lung tissue alteration. These results indicate that crocetin can provide protection against LPS-induced acute lung injury in mice.

1. Introduction

Acute lung injury and acute respiratory distress syndrome (ARDS) which is the severest form of injury, are the leading causes of morbidity and mortality in critically ill patients (Ni et al., 2010a, 2010b; Mutz et al., 2010; Ngamsri et al., 2010; Aman et al., 2011). Acute lung injury is characterised by the development of hypoxemia, damage to the alveolar capillary membrane barrier, pulmonary oedema, and the resultant respiratory failure (Pechulis et al., 2010; Matthay et al., 2003). Endotoxin or lipopolysaccharide (LPS) derived from gram-negative bacteria has been well recognised in the pathogenesis of acute lung injury. Experimental administration of LPS, both systemically and intratracheally, has been used to induce neutrophil infiltration and develop pulmonary inflammation in animal models (Beutler and Rietschel, 2003; Ingenito et al., 2001; Karmpaliotis et al., 2002; Kitamura et al., 2001). When the lung cells are exposed to LPS, the nuclear factor (NF)-κB is activated. NF-κB is a protein transcription factor that enhances the transcription of a variety of genes, including cytokines and growth factors, adhesion molecules, immunoreceptors, and acute-phase proteins (Blackwell and Christman, 1997).Upon activation by LPS, NF-κB is required for maximal transcription of numerous cytokines, including tumour necrosis factor-α (TNF-α), interleukin-6 (IL-6), and monocyte chemotactic protein-1 (MCP-1). Maximal transcription is very critical in the generation of acute lung injury (Bhatia and Moochhala, 2004; Agouridakis et al., 2002; Abraham et al., 2000). These cytokines and chemokines contribute to the vigorous acquisition of neutrophils in the lung. Therefore, acute lung injury is caused substantially by excessive neutrophil- and cytokine-mediated inflammation. Despite advancement in the understanding of the pathophysiology of acute lung injury and improved therapy methods, mortality rates related to acute lung injury have remained at approximately 40% (Sakr and Vincent, 2005; Ni et al., 2010a, 2010b).

Crocetin (>98%, Fig.1), a carotenoid compound, can be extracted from *Gardenia jasminoides* Ellis in the laboratory (Xi et al., 2005). Previous studies suggest that crocetin reduced mRNA and protein expression of inflammatory factor TNF-α in palmitate-treated adipocytes (Xi et al., 2007). Crocetin has been confirmed to inhibit reactive oxygen species production and inflammatory cascades towards amelioration of cardiac injury caused by haemorrhage/resuscitation (Yan et al., 2010). These previous studies, which were conducted in our lab, attest to the potential of crocetin as anti-inflammatory agent. However, the mechanisms by which crocetin influences inflammatory regulators have not been clarified. Thus, in the present study, the effects of crocetin on acute lung injury induced by LPS were investigated systematically. The involvement of NF-KB pathway in the development of acute lung injury was also studied. A discussion on whether crocetin could become a candidate therapeutic drug for acute lung injury is provided.

2. Materials and methods

2.1. Animals

Male and female ICR mice weighing 22–25 g were purchased from China Pharmaceutical University. All animals were housed in a room with temperature maintained at 20 ± 1 °C. Lights were provided from

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8:00 a.m. to 8:00 p.m. Animals were allowed free access to tap water and regular rodent chow. Rodent chow was withheld 8 h before the experiments. All the animal care and handling procedures were approved by the Institutional Animal Care and Use Committee of China Pharmaceutical University.

2.2. Reagents

Crocetin with purity greater than 98% was extracted from *Gardenia jasminoides Ellis* in the laboratory according to the procedures of an earlier study (Xi et al., 2005). The chemical structure of this compound is shown in Fig. 1. Lipopolysaccharide and Trizol reagents were purchased from Sigma (St. Louis, USA). Superoxide dismutase (SOD) and myeloperoxidase (MPO) determination kits were provided by Jiancheng Bioengineering Institute of Nanjing (Nanjing, China). RT-PCR kit which includes reverse transcriptase, dNTP mixture, Tag DNA polymerase, and RNAase inhibitor was obtained from Takara Bio Inc. (Otsu, Japan). TNF-α, IL-6, MCP-1, and GAPDH primers were purchased from Shanghai Bioengineering Ltd. (Shanghai, China). HRP-conjugated anti-rabbit IgG or anti-mouse IgG, antibodies for phospho-IκB and IκB were obtained from Santa Cruz Biotechnology (CA, USA). Enzyme-linked immunosorbent assay (ELISA) kit (catalogue NO. 2N909) for DNA-binding NF-κB p65 was purchased from Rapidbio Lab (CA, USA). Nuclear extract kit was obtained from NanJing KeyGen Biotech Ltd. (Nanjing, China). All other reagents were of analytical grade.

2.3. Experimental protocol for acute lung injury model

Acute lung injury was induced as follows: mice were anaesthetised with an intraperitoneal injection of 1.5 g/kg of urethane and randomly received a single intratracheal instillation with either 5 mg/kg LPS (LPS cohort, n = 30) or normal saline (control cohort, n = 20). Mice from LPS cohort were subsequently assigned to 3 groups. Mice from each of the first two groups (n = 10) were intragastrically administered with normal saline (vehicle group) and crocetin at 50 or 100 mg/kg (crocetin group). Mice from the control cohort were subsequently divided into two groups. Each group (n = 10) of mice was intragastrically administered with normal saline (vehicle control group) and 100 mg/kg crocetin (crocetin control group). Intragastrical administrations were performed 1, 12, 24, 36, and 48 h before LPS instillation, and 12 and 24 h after LPS instillation. After the 24 h post-instillation, lungs of mice were harvested for determination of MPO and SOD activities, lung water content, nuclear factor-kappa B (NF-κB) activity, and isolation of mRNA for RT-PCR assays, western blot analysis, and histological examination.

2.4. Lung water content

Twenty four hours after the intratracheal instillation of LPS or normal saline, lung tissues from upper left lobe were excised, sliced, and dried at 60 °C for 48 h. Subsequently, the lung water content was derived using the formula: W/D% = [(wet weight − dry weight) / dry weight] × 100.

2.5. Assay of MPO and SOD activities

Tissue samples were subjected to two further freeze–thaw cycles and centrifuged at 10,000 g for 10 min at 4 °C. The supernatant was assayed spectrophotometrically for MPO and SOD activities with assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). All procedures were performed according to manufacturer's instructions.

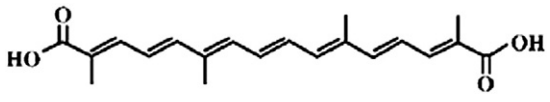

Fig. 1. Chemical structure of crocetin.

2.6. Histological examination

The lungs were fixed with 10% neutral formalin. Lung tissue was dehydrated with graded alcohol and subsequently embedded in paraffin in preparation for light microscopic examination. Sections, approximately 4 μm thick, were stained with haematoxylin and eosin. Light microscopy was performed to assess the general morphology. Histological examination was conducted by a histologist who was blinded to the treatment groups.

2.7. Real-time PCR for IL-6, MCP-1, and TNF-α mRNA expressions

Total RNA was extracted from the lungs at the indicated time points using Trizol® Reagent according to the manufacturer's instruction. After removal of potentially contaminating DNA with DNase I, cDNA was synthesised from 1 μg total RNA using a first strand cDNA synthesis kit (MBI). Equal quantities of cDNA from each animal were used for real-time PCR analysis of expression levels of TNF-α, IL-6,and MCP-1 using an Icycler (BioRad) with a SYBR Green qPCR kit (Invitrogen) as previously reported. The relative expression levels for each target gene were normalised by GAPDH and presented as fold increases vs. lung tissues from normal mice.

Oligonucleotide primer sequences for RT-PCR

Genes		Primer sequences (5′–3′)
TNF-α	Forward:	ACGGCATGGATCTCAAAGAC
	Reverse	GGTCACTGTCCCAGCATCTT
IL-6	Forward:	AGTTGCCTTCTTGGGACTGA
	Reverse	GCCACTCCTTCTGTGACTCC
MCP-1	Forward:	TGAGGTGGTTGTGGAAAAGG
	Reverse	CCTGCTGTTCACAGTTGCC
GAPDH	Forward:	TGCTCGAGATGTCATGAAGG
	Reverse	TTGCGCTCATCTTAGGCTTT

2.8. NF-κB activity and phospho-I-κB expression assay

Lung nuclear protein was extracted using a nuclear extract kit. NF-κB activity of the nuclear extracts was determined by ELISA p65 assay kit according to the manufacturer's instruction. Subsequently, 50 μl of standards or samples was added to the appropriate well of the antibody pre-coated microtitre plate. The plates were incubated for 1 h at 37 °C with mild agitation. After several washes, the antibody used to counter NF-κB p65 was detected to have bound to the complex following the addition of peroxidase-conjugated secondary antibody. Absorbance was measured using a spectrophotometer at 450 nm. The standard curve was generated by plotting the OD obtained for each of the six standard concentrations. The amount in each sample was used to determine the standard curve.

Phospho-I-κB expression was ascertained by the western blot technique. Nuclear extracts were prepared according to the improved method of Deryckere and Gannon. After protein quantisation using a Coomassie brilliant blue assay, 50 μg protein was boiled in loading buffer, resolved in 10% SDS-polyacrylamide gels, electrotransferred to nitrocellulose membranes, incubated with the monoclonal antibodies against phospho-I-κB (1:200) and I-κB (1:800) overnight, and probed with mouse secondary antibody (anti-rabbit IgG peroxidase conjugated, 1:8000). Relative content of target proteins was detected by chemiluminescence.

2.9. Data analysis

All data are presented as mean ± S.D. unless specified otherwise. The differences among groups were analysed using analysis of variance. P-values less than 0.05 were considered to be statistically significant.